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***In vitro* evaluation of the antiviral properties of Shilajit and investigation of its mechanisms of action**

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Running title: Antiviral activity of Shilajit

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25 **ABSTRACT**

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26 **ETHNOPHARMACOLOGICAL RELEVANCE:** Shilajit, a herbomineral substance exuded from
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57 rocks in steep mountainous regions, has been used for thousands of years by the Indian Ayurvedic
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78 and Siddha systems of traditional medicine to relieve ailments and enhance quality of life. Although
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9
29 a large number of therapeutic properties have been ascribed to Shilajit, its therapeutic potential is
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130 still largely unexplored by modern research and many of its claimed bioactivities lack scientific
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31 validation. The present study was undertaken to investigate the antiviral activity of Shilajit against a
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16
172 panel of viruses including herpes simplex type 1 and 2 (HSV-1, HSV-2), human cytomegalovirus
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1933 (HCMV), human respiratory syncytial virus (RSV), human rotavirus (HRV), and vesicular
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224 stomatitis virus (VSV).

23
245 **MATERIALS AND METHODS:** The antiviral activity of Shilajit was assayed *in vitro* by plaque
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276 reduction and virus yield assays and the major mechanism of action was investigated by virucidal
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297 and time-of-addition assays.

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32 **RESULTS:** Shilajit exhibited a dose-dependent inhibitory activity against HSV1, HSV2, HCMV,
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349 and RSV infectivity *in vitro* (EC₅₀ values: 31.08 µg/ml, 12.85 µg/ml, 34.54 µg/ml, and 30.35 µg/ml,
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3740 respectively), but was inactive against HRV and VSV. Humic acid, a constituent of Shilajit,
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3941 displayed the same spectrum of activity. Partial virus inactivation and interference with virus
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42 attachment were both found to contribute to the antiviral activity of Shilajit.

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443 **CONCLUSIONS:** The results of the present study demonstrate that Shilajit is endowed with broad,
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4744 yet specific, antiviral activity *in vitro* and constitutes a natural source of antiviral substances.
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4945 Further work remains to be done to assess its efficacy *in vivo*.

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47 **1. INTRODUCTION**

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28 The global impact of viral infections, the development of antiviral drug resistance, and the
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49 emergence of new viruses are all driving the incessant search for new compounds endowed with
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50 antiviral activity, with the aim of developing novel safe and effective antiviral treatments.

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51 In this context, natural products originating from botanical, animal or mineral sources traditionally
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11 used in ethnomedicine may provide leads for modern antiviral drug development once their
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13 pharmacological potential is verified by scientific investigation.

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This paper focuses on Shilajit, a herbomineral substance that has been used for thousands of years
by the Indian Ayurvedic and Siddha systems of traditional medicine to relieve ailments and enhance
the quality of life. It is a blackish-brown matter exuded from rocks in steep mountainous regions,
such as the Himalaya mountains between India and Nepal, as well as mountains in Russia, Tibet,
Afghanistan, and Norway (Agarwal et al., 2007). The chemical characterization of Shilajit has
revealed that it consists of three major components: 1) low and medium molecular weight non-
humic organic compounds; 2) medium and high molecular weight DCPs (dibenzo- α -pyrones-
chromoproteins), containing trace metal ions and coloring matter such as carotenoids and indigoids;
and 3) metallo-humates, like humic acids, fulvic acids and fusims with dibenzo- α -pyrones in their
core nuclei (Ghosal 2006). Shilajit has been considered a panacea by many traditional systems of
oriental medicine, which have ascribed a vast array of therapeutic properties to this natural
substance (Agarwal et al., 2007, Wilson et al., 2011). Preclinical studies have pointed to the
potential use of Shilajit in various pathological conditions due to its numerous properties/actions,
which include: antiulcerogenic properties, antioxidant properties, complement activator in the
immune system and immunomodulator, antidiabetic properties, anxiolytic and antistress properties,
anti-inflammatory and anti-allergic properties, and memory and learning enhancer (Agarwal et al.,
2007, Wilson et al., 2011). However, a recent systematic review of early studies revealed that the
full therapeutic potential of Shilajit is still largely unexplored by modern research and many of its
claimed bioactivities lack scientific validation and remain unproven (Wilson et al., 2011). The

73 antiviral potential of Shilajit has yet to be explored, with only its anti-HIV action having received
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24 research attention to date (Ghosal, 2006, Gupta et al., 2010, Rege et al., 2012). The antiviral activity
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5 of one of its components, humic acid has been partially explored (Klöcking et al., 2005).
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76 The present study was undertaken to investigate the antiviral activity of Shilajit against a panel of
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85 **2. MATERIALS AND METHODS.**

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3 86 **2.1 Compounds.** Shilajit was purchased from Dekha Herbals (Lalitpur, Nepal). On the basis of the
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5 87 certificate of analysis provided by the manufacturer, humic acid and fulvic acid represents the 9.5%
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7 88 and the 26.6 % of Shilajit composition respectively. The following components are also present: 4-
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9 89 methyl catechol, benzoic acid, benzamide, ethyl benzoate, hydrobenzoin, carboxy ethane,
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12 90 ammonium benzoate, homocatechol, orcinol, β eudesmol, isodamascol, juniper camphor, and
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14 91 stearyl. Shilajit was dissolved in bi-distilled sterile water to make a 25mg/ml stock solution prior
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17 92 to each experiment. Humic acid, heparin, acyclovir and foscarnet were purchased from Sigma (St.
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19 93 Louis, Mo., USA).

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23 94 **2.2 Cells.** African green monkey fibroblastoid kidney cells (Vero, ATCC CCL-81), human
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25 95 epithelial cells (Hep-2) (ATCC CCL-23), A549 (ATCC CCL-185), and African green monkey
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28 96 kidney epithelial (MA-104) cells (ATCC CRL-2378.1) were grown as monolayers in Eagle's
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30 97 minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat
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33 98 inactivated fetal calf serum (FCS) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva
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35 99 Biolabs GmbH, Berlin, Germany). Low-passage human embryonic lung fibroblasts (HELFs) were
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38 100 grown as monolayers in Eagle's minimal essential medium (Gibco-BRL) in the same conditions as
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41 101 described above with the addition of 1mM sodium pyruvate.

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45 103 **2.3 Viruses.** Clinical isolates of HSV-1 and HSV-2 were kindly provided by Prof. M. Pistello,
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47 104 University of Pisa, Italy. HSV-1 and HSV-2 strains were propagated and titrated by plaque assay on
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49
50 105 Vero cells. A HSV-2 strain with phenotypic resistance to acyclovir was generated by serial passage
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52 106 in the presence of increasing concentrations of acyclovir, as previously described (Donalisio et al.,
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55 107 2013). HCMV strain Towne was kindly provided by Prof. W. Brune, Heinrich Pette Institut,
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57 108 Hamburg, Germany; it was propagated and titrated by plaque assay on HELF cells. RSV strain A2
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59 109 (ATCC VR-1540) was propagated in Hep-2 and titrated by the indirect immunoperoxidase staining
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110 procedure using an RSV monoclonal antibody (Ab35958; Abcam, Cambridge, United Kingdom), as
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111 described previously (Donalisio et al., 2012). Human rotavirus strain Wa (ATCC VR-2018) was
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112 activated with 5 µg/mL porcine pancreatic trypsin type IX (Sigma, St. Louis, Mo.) for 30 minutes at
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113 37°C and propagated in MA104 cells using MEM containing 0.5 µg trypsin per mL, as described
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114 previously (Graham et al., 2004). Virus stocks were maintained at -80 °C.

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116 **2.4 Cell viability.** Cell viability was measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-
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117 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Confluent cell cultures seeded in
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118 96-well plates were incubated with different concentrations of Shilajit or humic acid in triplicate
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119 under the same experimental conditions described for the antiviral assays. Cell viability was
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120 determined using the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) according
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121 to the manufacturer's instructions. Absorbances were measured using a Microplate Reader (Model
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122 680, BIORAD) at 490 nm. The effect on cell viability at different concentrations of the compound
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123 was expressed as a percentage, by comparing absorbances of treated cells with those of cells
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124 incubated with culture medium alone. The 50% cytotoxic concentrations (CC₅₀) and 95%
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125 confidence intervals (CIs) were determined using Prism software (Graph-Pad Software, San Diego,
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126 CA).

127 **2.5 HSV inhibition assays.** The effect of Shilajit on HSV infection was evaluated by plaque
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128 reduction assay. Vero cells were pre-plated 24 h in advance in 24-well plates at a density of 10 x
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129 10⁴ cells. Increasing concentrations of compounds were added to cells for 2 h; a mixture of the
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130 compound plus HSV-1, HSV-2, or acyclovir resistant HSV-2 (MOI 0.0003 pfu/cell) was
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131 subsequently added to the cells, which were then incubated at 37 °C for 2 h. The virus inoculum
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132 was then removed and the cells washed and overlaid with a medium containing 1.2%
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133 methylcellulose (Sigma) and serial dilutions of Shilajit or humic acid. After further incubation at
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134 37 °C for 24 h (HSV-2) or 48 h (HSV-1), cells were fixed and stained with 0.1% crystal violet in
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135 20% ethanol and viral plaques counted. The effective concentration producing 50% reduction in
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136 plaque formation (EC₅₀) was determined using Prism software by comparing drug-treated with
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137 untreated wells. The selectivity index (SI) was calculated by dividing the CC₅₀ by the EC₅₀ value.
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8 **2.6 HCMV inhibition assay.** HELF cells were pre-plated in a 96-well plate. The following day
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10 serial dilutions of Shilajit or humic acid were added to cells and incubated for 2 h at 37°C. Virus
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139 (MOI 0.005) and compound were then added and the cells incubated for a further 2 h; monolayers
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140 were then washed and overlaid with 1.2% methylcellulose medium supplemented with 3% FCS and
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141 1mM sodium pyruvate. After five days incubation, cells were fixed with cold methanol and acetone
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143 for 1 min and subjected to HCMV-specific immunostaining using an anti-HCMV IEA monoclonal
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144 antibody (11-003; Argene, Verniolle, France).
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26 **2.7 RSV inhibition assay.** Serial dilutions of the compound were added to A549 cells grown as
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28 monolayers in a 96-well plate and incubated for 2 hours at 37°C. Mixtures of the compounds and
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145 virus (MOI 0.01) were then added to the cells and incubated for a further 3 hr at 37°C to allow viral
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147 adsorption; the monolayers were then washed and overlaid with 1.2% methylcellulose medium
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148 supplemented with Shilajit or humic acid. Three days post-infection, cells were fixed with cold
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149 methanol and acetone for 1 min and subjected to RSV-specific immunostaining. Immunostained
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150 plaques were counted, and the percent inhibition of virus infectivity determined by comparing the
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151 number of plaques in treated wells with the number in untreated control wells.
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46 **2.8 Rotavirus inhibition assay.** To assess the compound's ability to inhibit rotavirus infectivity,
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48 assays were carried out using confluent MA104 cell monolayers plated in 96-well trays. Virus
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50 infectivity was activated by adding 5µg porcine trypsin (Sigma)/mL for 30 minutes at 37°C. Cells
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52 were pre-treated for 1 hours with serial dilutions of Shilajit and subsequently activated virus (MOI
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54 0.02 pfu/cell) and Shilajit were added to cells for 1 h at 37°C; cells were then washed and fresh
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56 medium containing Shilajit added. After 16 h, cells were fixed with cold acetone-methanol and viral
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59 titers were determined by indirect immunostaining using the monoclonal antibody mab-0036
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160 (specific for human 41 kDa inner capsid protein - VP6 - of Rotavirus) purchased from Covalab
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161 (Villeurbanne, France) and the UltraTech HRP Streptavidin-Biotin Detection System (Beckman
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162 Coulter).

163 **2.9 VSV inhibition assay.** Vero cells were pre-plated 24 h in advance in 24-well plates at a density
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164 of 10×10^4 cells/well. Increasing concentrations of compound were incubated with cells for 2 h at
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165 37°C, then a mixture of VSV (MOI 0.0005 pfu/cell) and compound added and incubated for a
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166 further 2 h at at 37°C. The virus inoculum was then removed and the cells washed and then overlaid
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167 with a medium containing 1.2% methylcellulose (Sigma) and serial dilutions of Shilajit. After 24 h
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168 of incubation at 37 °C, cells were fixed and stained with 0.1% crystal violet in 20% ethanol and
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169 viral plaques were counted.

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171 **2.10 Time-of-addition assay.** Serial dilutions of Shilajit were added to cells: 2 h before infection,
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172 during infection, or post infection. After the incubation time, viral plaques were counted.
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174 **2.11 Investigation of the mechanism of action.** Vero and A549 cells were plated as described
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175 above and subjected to 3 different kinds of assay:
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176 **2.11.1 Attachment assay.** Serial dilutions of Shilajit were mixed with HSV2 or RSV, added
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177 to cooled cells, and incubated for 2 h at 4°C to ensure viral attachment but not entry. After
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178 two gentle washes, cells were overlaid with 1.2% methylcellulose medium and shifted to
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179 37°C for 24 or 72 h. At the end of incubation, plaques were counted.
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180 **2.11.2 Entry assay.** For entry assays, HSV-2 and RSV at a MOI of 0.001pfu/cell and 0.01
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181 pfu/cell, respectively, were adsorbed for 2 h at 4°C on pre-chilled confluent Vero or A549
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182 cells. Cells were then washed with cold MEM three times to remove unbound virus, treated
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183 with different concentrations of extract, and incubated for 3 h at 37°C. Unpenetrated viruses
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184 were inactivated with acidic glycine for 2 min at room temperature, as previously
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185 described (Shogan et al., 2006). Cells were then washed with warm medium 3 times and
186 treated as described above for plaque reduction assay.

187 **2.11.3 Virus inactivation assay.** Approximately 10^5 PFU of HSV2 or 10^4 PFU of RSV plus
188 100 µg/ml of Shilajit were added to MEM and mixed in a total volume of 100 µl. The virus-
189 compound mixtures were incubated for 2 h at 37 °C or 4°C then diluted serially to the non-
190 inhibitory concentration of test compound; the residual viral infectivity was determined by
191 viral plaque assay.

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193 **2.12 Virus yield reduction assay.** For the virus yield reduction assay, cells were pre-treated with
194 Shilajit for 2 h at 37°C then infected in duplicate with HSV-2 at an MOI of 0.007 pfu/cell or RSV at
195 an of MOI 0.005 pfu/cell in presence of Shilajit; following virus adsorption (2 or 3 h at 37°C), the
196 viral inoculum was removed and cultures were exposed to Shilajit and incubated until control
197 cultures displayed extensive cytopathology. Supernatants and cells were harvested and cell-free
198 virus infectivity titers were determined in duplicate by plaque assay in Vero or A549 cell
199 monolayers. The same experiment was also conducted with Shilajit only being added post-
200 infection. Percent inhibition was determined by comparing the titer measured in the presence of the
201 compounds to that measured in untreated wells.

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203 **2.13 Data analysis.** All results are presented as the mean values from three independent
204 experiments. The EC_{50} values for inhibition curves were calculated by regression analysis using the
205 software GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, U.S.A.) by
206 fitting a variable slope-sigmoidal dose–response curve.

208 **3. RESULTS AND DISCUSSION.**

209 To test whether Shilajit can affect HSV-1, HSV-2, HCMV, RSV, human rotavirus, and VSV
 210 infectivity *in vitro*, we used a complete protection assay in which the compound was added to the
 211 cell culture before, during, and after the infection. Shilajit exhibited dose-dependent inhibitory
 212 activity against HSV1, HSV2, HCMV, and RSV infectivity; the EC₅₀ values are shown in Table 1
 213 in comparison with those of reference antiviral compounds (i.e. acyclovir for HSV, foscarnet for
 214 HCMV, and heparin for RSV). Of note, Shilajit did not affect cell viability at concentrations as high
 215 as 1500 µg/mL, demonstrating that the antiviral effect was not a consequence of cytotoxicity.
 216 Although Shilajit proved active against three viruses belonging to the *Herpesviridae* family (i.e.
 217 HSV-1, HSV-2, and HCMV) and one of the *Paramyxoviridae* family (RSV), the lack of inhibitory
 218 activity against rotavirus (*Reoviridae*) and VSV (*Rhabdoviridae*) indicates that the spectrum of
 219 Shilajit's activity is specific to definite viruses.

221 **Table 1. Antiviral activity of Shilajit and of reference compounds**

	virus	EC₅₀ µg/mL* (95% C.I.)**	SI***
Shilajit	HSV-1	31.08 (23.64-40.87)	> 48.26
	HSV-2 acyclovir sensitive strain	12.85 (6.60-25.02)	> 116.7
	HSV-2 acyclovir resistant strain	14.39 (10.18-20.34)	> 104.24
	HCMV	34.54 (23.96-49.79)	> 43.43
	RSV	30.35 (18.41-50.04)	> 49.42
	Rotavirus	-	-
	VSV	-	-
Acyclovir	HSV-1	0.037 (0.025-0.055)	>2702

	HSV-2 acyclovir sensitive strain	0.14 (0.098-0.21)	>714
	HSV-2 acyclovir resistant strain	71.84 (41.3-101.4)	>1.41
Foscarnet	HCMV	5.17 (3.91-6.93)	>24.5
Heparin	RSV	0.048 (0.028-0.082)	> 685

* EC₅₀: 50% effective inhibitory concentration

** 95% CI: 95% confidence interval

*** SI: selectivity index

Moreover, as humic acid is a major component of Shilajit, we tested its antiviral activity. The results presented in Table 2 demonstrate that humic acid is endowed with antiviral activity against HSV-1, HSV-2 HCMV, and RSV with EC₅₀ values that are lower than those calculated for Shilajit, with HCMV as the only exception. These results indicate that humic acid may contribute to the overall antiviral activity of Shilajit.

Table 2. Antiviral activity of humic acid

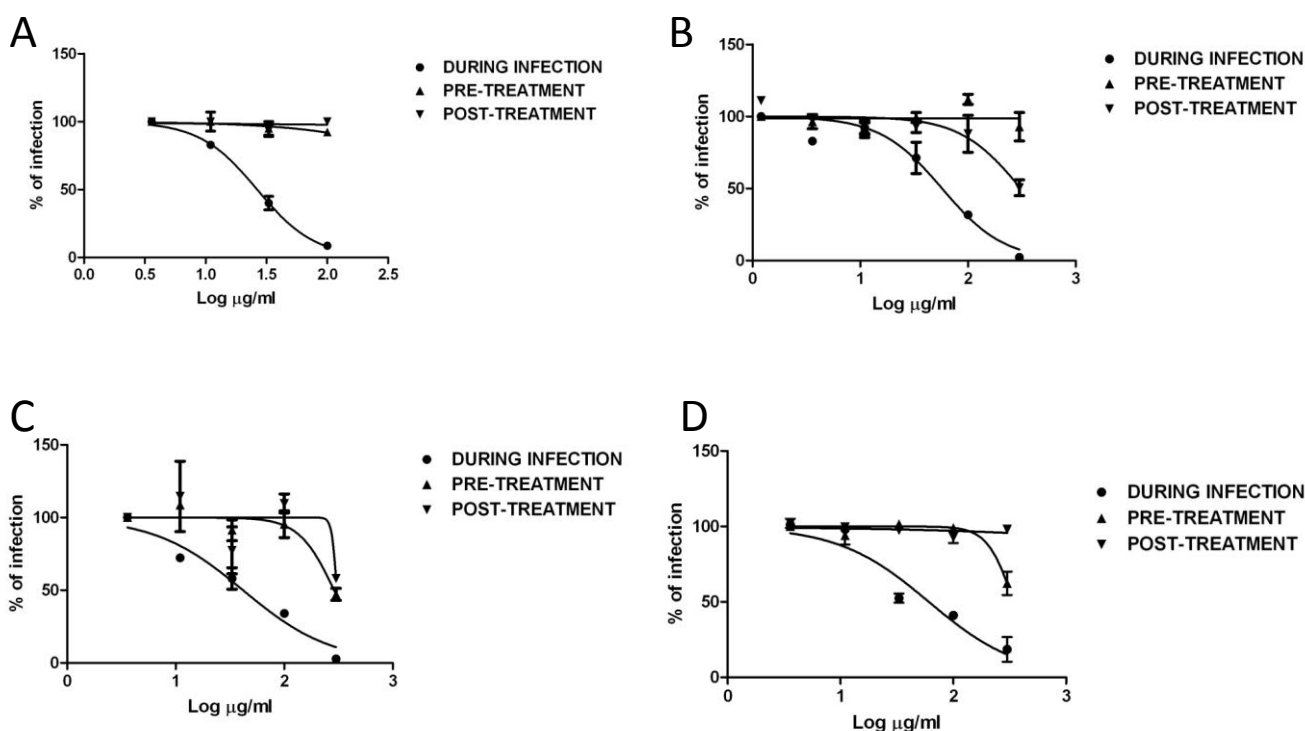
	virus	EC ₅₀ µg/mL* (95% C.I.)**	SI***
Humic Acid	HSV-1	4.83 (2.87-8.13)	>311
	HSV-2 acyclovir sensitive strain	2.41 (1.89-3.08)	>622
	HCMV	38.3 (33.8-43.5)	24.6
	RSV	12.34 (10.41-14.63)	>39.2

* EC₅₀: 50% inhibitory concentration

** 95% CI: 95% confidence interval

*** SI: selectivity index

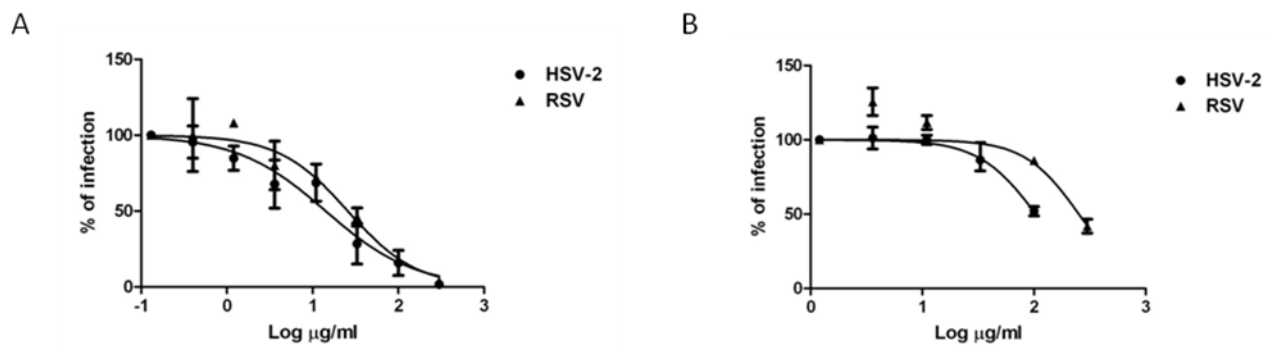
237 To investigate Shilajit's mechanism of action we performed a series of time-of-addition assays, in
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 238 which the compound was added to the cells only before, or during, or after infection. As shown in
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 239 Figure 1, Shilajit exerted dose-dependent inhibitory activity only when added during infection with
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 240 EC₅₀ values of 26.74 μg/mL for HSV-2, 57.58 μg/mL for HSV-1, 41.73 μg/ml for HCMV, and
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 241 61.44 μg/mL for RSV. By contrast, inhibition was limited to the higher doses tested or was absent
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 242 altogether in the pre-treatment or post-treatment assays, thus EC₅₀ values could not be determined.
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 244 **Fig.1 Time-of-addition assay.** Vero (A-B), HELF (C), or A549 (D) cells were treated with Shilajit prior to virus
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 245 infection (pre-treatment), during the infection period (during infection), or after infection (post-treatment) with HSV-2
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 246 (A), HSV-1 (B), HCMV (C), or RSV (D). Data are presented as % of control. Values are means ± SEM of three
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 247 independent experiments.
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 250 Results from the time-of-addition assays suggest that Shilajit may either target the early steps of the
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 251 virus replicative cycle (i.e. virus attachment or entry into cells) or act as a virucide by irreversibly
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 252 inactivating the viral particles. To investigate these hypotheses, HSV-2 and RSV were chosen for
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253 further experiments as representative viruses of the *Herpesviridae* and *Paramyxoviridae* virus
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 254 families, respectively. First, we carried out an attachment assay, an experimental condition in which
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 255 the virus is allowed to bind to the surface of the host cells, in the presence or absence of Shilajit, but
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 6 not undergo cell entry. As shown in Fig. 2A, Shilajit inhibited HSV-2 and RSV infectivity with
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 256 EC₅₀ of 14.20 µg/ml and 25.70 µg/ml, respectively; values that are comparable to those reported in
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 257 Table 1. This result indicates that the antiviral activity of Shilajit depends, at least in part, on its
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 258 capacity to prevent the attachment of the viruses to the cell surface. By contrast, when the antiviral
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 259 activity of Shilajit was tested using an entry assay in which Shilajit was added immediately after
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 260 virus attachment to assess its ability to prevent entry, only a weak inhibition was observed at high
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 261 doses (Fig. 2B).
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 262 doses (Fig. 2B).



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 264 **Fig.2 Attachment and entry assay.** In the attachment assay (A), Vero cells were infected with HSV-2 and A549 cells
 265 were infected with RSV in presence of serial dilutions of Shilajit; inoculated cultures were then kept for 2 h at 4°C to
 266 allow virus attachment but not entry, and then tested in plaque reduction assays. In the entry assay (B), cells were
 267 infected in the absence of Shilajit and once again kept at 4°C for 2 h to allow virus attachment; serial dilutions of the
 268 compound were added to washed cells and the temperature then shifted to 37°C to allow entry. After a single wash with
 269 acidic glycine to remove virus particles from the cell surface, cells were overlaid with medium containing
 270 methylcellulose. Data are presented as % infectivity of control. Values are mean ± SEM of three separate
 271 determinations.

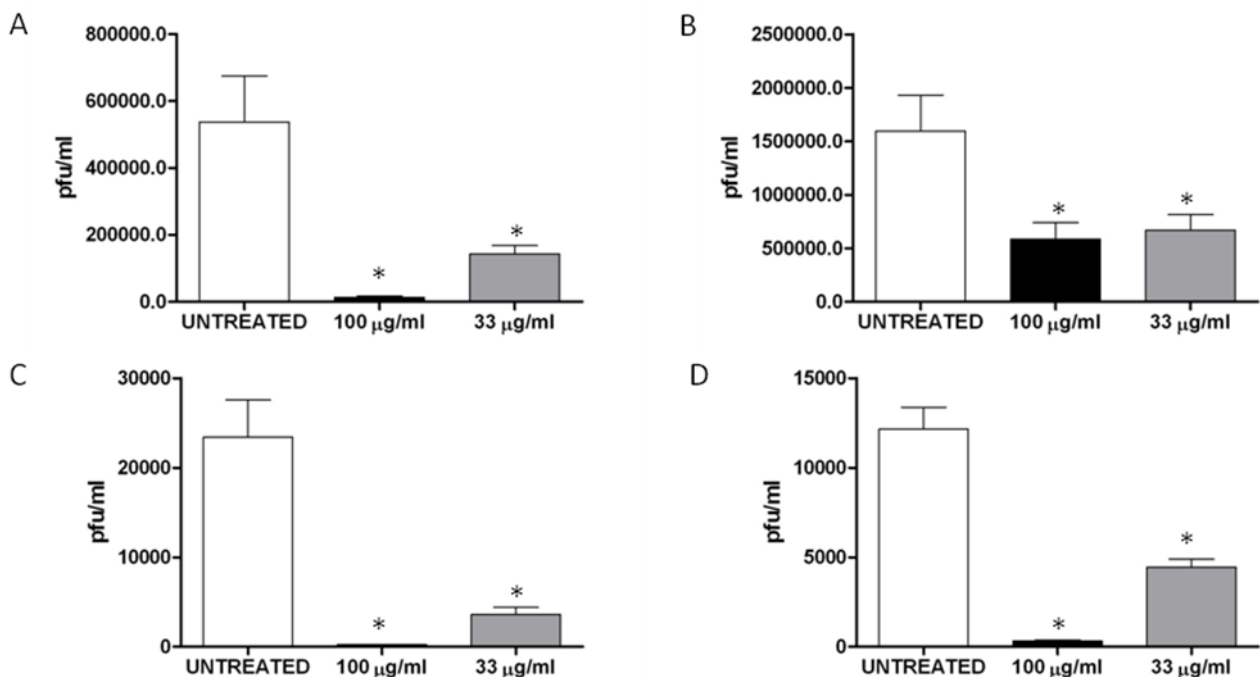
272
 273 To explore the possibility that Shilajit also exerts direct virus-inactivating activity, a virucidal assay
 274 was performed using the effective concentration that reduced virus infection almost completely

275 (EC₉₀) in the standard assay. To this end, HSV-2 and RSV aliquots were incubated with 100 µg/ml
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276 Shilajit at 4°C or 37°C for 2 h. After incubation, the samples were titrated on Vero or A549 cells
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277 using high dilutions at which Shilajit was no longer active as an antiviral. When the incubations
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278 were carried out at 37°C, Shilajit produced a significant loss of both HSV-2 (71.2% inhibition) and
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279 RSV (74.4% inhibition) titers, although the treatment did not completely abrogate infectivity. This
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280 result indicates that partial virus inactivation contributes to the overall antiviral activity of Shilajit.
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281 By contrast, no virucidal activity was observed when the incubation was carried out at 4°C. This
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282 latter result rules out the possibility that the antiviral activity observed in the attachment assay,
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283 performed at 4°C, was due to a direct virus inactivation.
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284 Some preliminary conclusions can be drawn on the main mode of antiviral action of Shilajit. Time-
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285 of-addition and virucidal assays indicate that the inhibitory effect mainly depends on the capacity of
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286 Shilajit to interact with the virus particles, rather than with cell components, thereby preventing
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287 virus attachment to the cell surface. This view is supported by several lines of evidence. First, cells
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288 pre-treated with Shilajit remained fully susceptible to virus infection, thus excluding the possibility
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289 that this compound could act by stably interacting with a cellular component(s) and thereby prevent
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290 its(their) interaction with viral glycoproteins. Second, the results of attachment and entry assays
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291 demonstrate that Shilajit blocks the adsorption of HSV-2 and RSV virions to the cell surface, but
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292 not entry. Third, pre-incubation of HSV-2 and RSV virions with Shilajit resulted in loss of
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293 infectivity, suggesting that both partial virus inactivation and interference with virus attachment
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294 contribute to the antiviral activity.
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296 To complete the *in vitro* analysis of the antiviral potential of Shilajit against HSV-2 and RSV, the
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297 compound was also evaluated by viral yield reduction assay – a more stringent test which allows
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298 multiple cycles of viral replication to occur before measuring the production of infectious viruses.
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299 The assay was conducted under two different conditions: 1) Shilajit was added before, during, and
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300 after infection (Fig 3 A-C); and 2) only after infection (Fig 3 B-D). In both cases, Shilajit was

301 active, with the strongest inhibition occurring in the first condition. This result indicates that besides
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302 preventing initial viral infection, Shilajit can also limit ongoing infection *in vitro*. This feature
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303 might be relevant for *in vivo* infections, characterized by the continuous release of virions by
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304 infected cells that promptly interact with neighboring cells, often resulting in direct cell-to-cell
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305 spread and syncytia formation. Of note, an acyclovir-resistant HSV-2 strain was as susceptible to
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306 Shilajit as the acyclovir-sensitive strain (Table 1), suggesting that the mode of antiviral action is
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307 different from that of acyclovir - a widely used inhibitor of the viral DNA polymerase. This latter
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308 feature, along with Shilajit's low cytotoxicity and favorable selectivity index make this natural
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309 compound a promising starting material for bioguided fractionation, with the aim of identifying
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310 anti-HSV-2 or anti-RSV compounds with novel mechanisms of action, which might also be used
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311 against acyclovir-resistant HSV-2 strains.
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312 Overall, the results of the present study demonstrate that Shilajit constitutes a natural source of
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313 antiviral substances endowed with broad, yet specific, antiviral activity *in vitro* and are in line with
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314 the use of Shilajit as antiseptic and germicide in traditional medicine (Wilson et al., 2011). In this
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315 work, we demonstrated that humic acid, a major component of Shilajit, is endowed with antiviral
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316 activity, however, further work remains to be done to isolate the active constituents and elucidate
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317 their mechanism(s) of action.
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319 **Fig. 3 Viral yield reduction.** Panels A and B refer to Vero cells infected with HSV-2; while panels C and D refer to
320 Hep-2 cells infected with RSV. Shilajit was added before, during, and after infection (A, C), or only after infection (B,
321 D). When the cytopathic effect involved the whole monolayer of untreated cells, the supernatant was harvested and
322 titrated. Plaques were counted and percent infection calculated by comparing treated with untreated (control) wells.
323 Viral titers (expressed as PFU/ml) are shown as means plus SEM for three independent
324 experiments. *, P<0.05.

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11
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386 **FIGURE CAPTIONS**

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5 **Fig.1 Time-of-addition assay.** Vero (A-B), HELF (C), or A549 (D) cells were treated with Shilajit
6 prior to virus infection (pre-treatment), during the infection period (during infection), or after
7 infection (post-treatment) with HSV-2 (A), HSV-1 (B), HCMV (C), or RSV (D). Data are
8 presented as % of control. Values are means \pm SEM of three independent experiments.

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15 **Fig.2 Attachment and entry assay.** In the attachment assay (A), Vero cells were infected with
16 HSV-2 and A549 cells were infected with RSV in presence of serial dilutions of Shilajit; inoculated
17 cultures were then kept for 2 h at 4°C to allow virus attachment but not entry, and then tested in
18 plaque reduction assays. In the entry assay (B), cells were infected in the absence of Shilajit and
19 once again kept at 4°C for 2 h to allow virus attachment; serial dilutions of the compound were
20 added to washed cells and the temperature then shifted to 37°C to allow entry. After a single wash
21 with acidic glycine to remove virus particles from the cell surface, cells were overlaid with medium
22 containing methylcellulose. Data are presented as % infectivity of control. Values are mean \pm SEM
23 of three separate determinations.

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27 **Fig. 3 Viral yield reduction.** Panels A and B refer to Vero cells infected with HSV-2; while panels
28 C and D refer to Hep-2 cells infected with RSV. Shilajit was added before, during, and after
29 infection (A, C), or only after infection (B, D). When the cytopathic effect involved the whole
30 monolayer of untreated cells, the supernatant was harvested and titrated. Plaques were counted and
31 percent infection calculated by comparing treated with untreated (control) wells. Viral titers
32 (expressed as PFU/ml) are shown as means plus SEM for three independent experiments. *, P<0.05.

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Table 1. Antiviral activity of Shilajit and of reference compounds

	virus	EC₅₀ µg/mL* (95% C.I.)**	SI***
Shilajit	HSV-1	31.08 (23.64-40.87)	> 48.26
	HSV-2 acyclovir sensitive strain	12.85 (6.60-25.02)	> 116.7
	HSV-2 acyclovir resistant strain	14.39 (10.18-20.34)	> 104.24
	HCMV	34.54 (23.96-49.79)	> 43.43
	RSV	30.35 (18.41-50.04)	> 49.42
	Rotavirus	-	-
	VSV	-	-
Acyclovir	HSV-1	0.037 (0.025-0.055)	>2702
	HSV-2 acyclovir sensitive strain	0.14 (0.098-0.21)	>714
	HSV-2 acyclovir resistant strain	71.84 (41.3-101.4)	>1.41
Foscarnet	HCMV	5.17 (3.91-6.93)	>24.5
Heparin	RSV	0.048 (0.028-0.082)	> 685

* EC₅₀: 50% effective inhibitory concentration

** 95% CI: 95% confidence interval

*** SI: selectivity index

Table 2. Antiviral activity of humic acid

	virus	EC₅₀ µg/mL* (95% C.I.)**	SI***
Humic Acid	HSV-1	4.83 (2.87-8.13)	>311
	HSV-2 acyclovir sensitive strain	2.41 (1.89-3.08)	>622
	HCMV	38.3 (33.8-43.5)	24.6
	RSV	12.34 (10.41-14.63)	>39.2

* EC₅₀: 50% inhibitory concentration

** 95% CI: 95% confidence interval

*** SI: selectivity index

Figure 1
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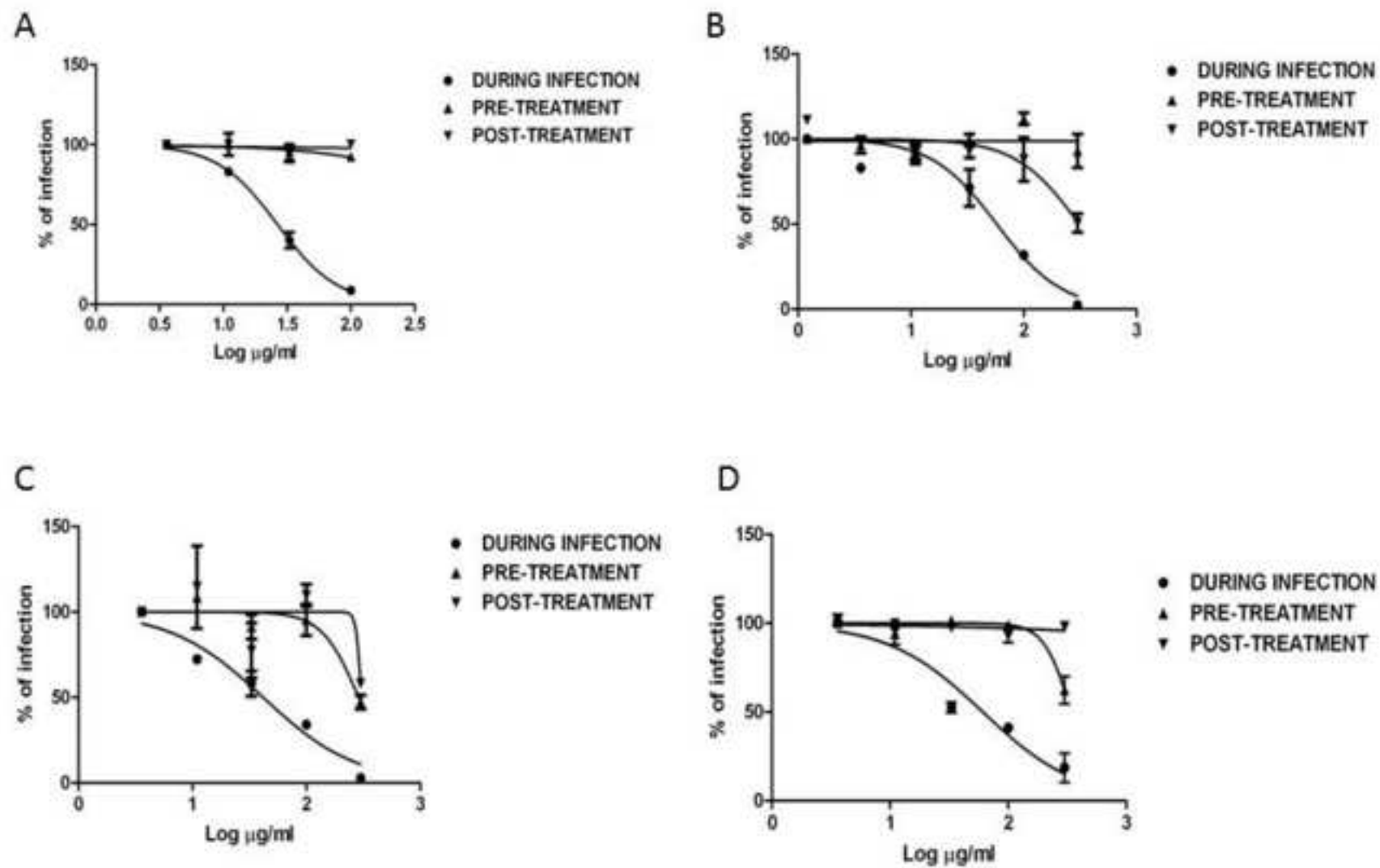
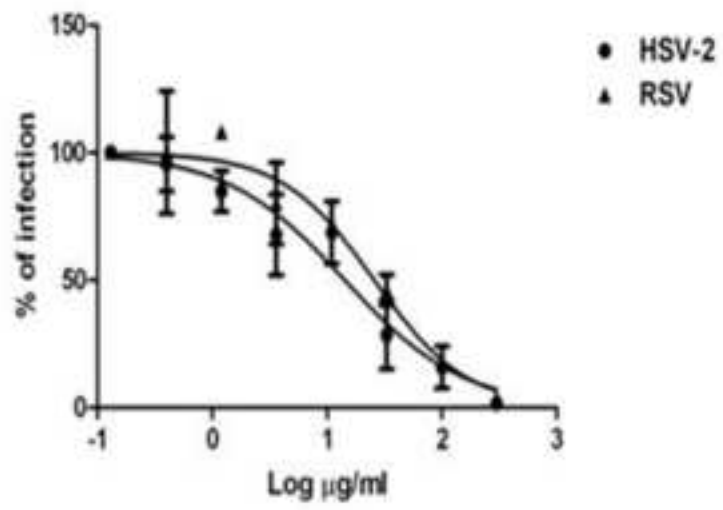


Figure 2
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A



B

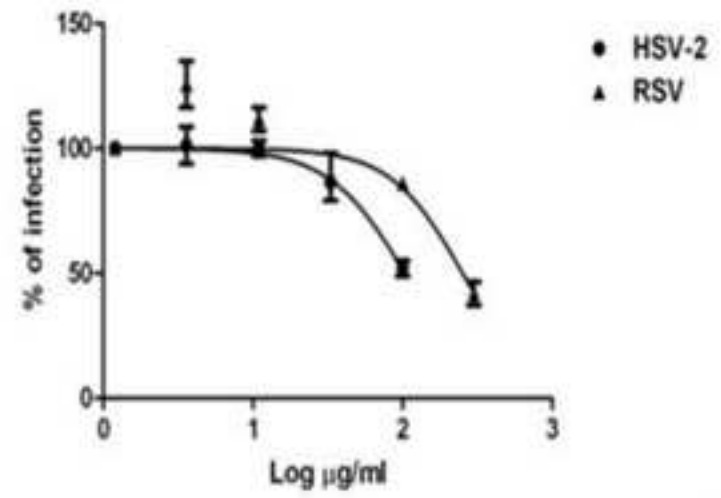


Figure 3
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